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Identification and molecular characterization of groundnut leaf miner in Uganda

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Abstract**—** *Although the leaf miner attacking groundnut in Africa has been widely reported as Aproaerema modicella (Deventer), a common groundnut (Arachis hypogaea L.) and soya bean (Glycine max (L.) Merr.), a Pest in Indo-Asian countries, a proper taxonomic identification of the pest has not been completed. A survey for species diversity of the pest was conducted on groundnut, the common host crops for leafminer species in Uganda, across 100 groundnuts farmers' fields in four agro ecological zones during the 2016-2017 growing season. 80 specimens comprising 40 larvae, 25 pupae and 15 moths of what was thought to be A. modicella (all from groundnut) were collected from the ten survey sites, and their mitochondrial DNA (mtDNA) COI were sequenced and compared with those from the BOLD gene bank. Infestation by GLM was observed on all groundnut fields sampled with Eastern Uganda being the hot ecological spot especially Namutumba district. The mtDNA COI from all specimens of the pest, matched 100% with the sequences in BOLD belonging to Aproaerema simplexella PS1, a species occurring in Australia, and known as the soya bean moth in that country. There was very little genetic diversity between and within the populations from the ten sites, which suggested that the populations were maternally of the same origin. Conclusively, this study like other studies elsewhere in Africa confirm to the fact that the leafminer attacking groundnuts and other crops such as soya bean was A. simplexella PS1 (100% match on the BOLD system), native to Australia, which suggested that Australia may be the origin of the pest not Indo-Asian countries.*

Keywords**— *Arachis hypogaea, Aproaerema modicella, mitochondrial DNA.***

I. INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is the second most important legume after beans (Phaseolus *vulgaris)* in both nutritional and economic empowerment of communities in Uganda. It's grown primarily for high quality edible oil (36-54% on dry matter basis) and easily digestible proteins (20-50%) in its seeds (D. okello *et al*, 2010). It is cultivated worldwide in tropical, sub-tropical and warm temperature areas located between 400N to 400S with world production of 36.9 million tonnes from an area of 25.2 million ha. Africa accounts for 40% of the global area planted to groundnuts, with only 26% production. The highest average yields were observed in Southern Africa and the lowest in East Africa (ICRISAT 2012). In 2006, the average groundnut yield recorded in Sub Saharan Africa was 980 kg/ha, considerably less than the world average of 1,690 kg/ha (Bucheyeki *et al.* 2008). The production of these crops on the continent is seriously threatened by a leaf miner, widely reported as Aproaerema modicella (Deventer) (Lepidoptera: Gelechiidae) (Kenis and Cugala, 2006). First noticed as a serious pest on groundnut in 1998 in Uganda and described as groundnut

leaf miner (GLM), the pest has raised considerable alarm and concern in the groundnut production zones of Uganda (Epieru, 2004).

In Uganda, GLM is known to be present in all groundnut producing areas of the country (Epieru 2004). However, the epidemic of GLM on groundnut in Uganda is sporadic and its severity appears to vary from place to place and from year to year, making it extremely difficult to predict. As a new pest, not much information is available on its ecology and ecophysiology that might help to predict its incidence and outbreaks.

The identity of the GLM in Africa, including Uganda, has generally been assumed to be A. modicella (Subrahmanyam *et al.,* 2000; Page *et al.,* 2000; Du Plessis, 2002; Munyuli *et al.,* 2003; Epieru, 2004; Kenis and Cugala, 2006) although Shanower *et al.* (1993) has hinted that it might be a different species. Since no proper taxonomic identification has been done on this new pest with no/little information characterizing this pest in Uganda and hence this study, its more probable that adoption of the name A. modicella was based on morphological characteristics of the larvae and adults and on crop damage symptoms that are similar to those of A. modicella in addition to strong prevalence of the pest on groundnut elsewhere in the world (Du Plessis, 2002, 2003; Kenis and Cugala, 2006). Van der Walt et al. (2008) examined the gonads of the female and male larvae of the GLM specimens collected in South Africa, and concluded that they were similar to those reported for A. modicella in Asia by Shanower et al. (1993), which reinforced the assumption that the pest was A. modicella. Because of its sudden appearance, the GLM occurring in Africa is thought to be a recent invasion from the IndoAsian continent (Kenis and Cugala, 2006) where A. modicella is native and seriously infests groundnut and soybean (Shanower *et al.,* 1993). Whilst this is possible, the pest may have evolved and spread within Africa.

Whereas morphological studies have been the keystone of insect pest identification in the past, and continue to be in the present, modern molecular techniques offer complementary, faster and more precise options for species identification (Scheffer, 2000), and is especially useful in differentiating related species that share similar morphological characteristics.

In addition, molecular techniques, e.g. DNA finger printing, especially those involving the mitochondrial DNA (mtDNA), are more reliable in pinpointing or tracing the geographical origin/links of pests and their paths of spread, (Scheffer, 2000; Simmons and Scheffer, 2004). There were two objectives in the present study. The first objective was to identify the species of leafminer attacking groundnuts in Uganda.t and the second objective was to determine its inter and intra-population genetic diversity by analysing in both cases the mtDNA CO1 gene of specimens collected from widely separated sites.

II. MATERIALS AND METHODS

Sampling

Groundnut leaf miner samples (both adult and larvae) were collected from ten agro ecological zones in Uganda. All larvae were preserved in 70% ethanol then brought to the *icipe* Molecular Pathology Lab in the Arthropod Pathology Unit for processing. Morphological characteristics were documented using a Leica EZD stereomicroscope (Leica Microsystems (UK) Ltd) then the samples were immediately preserved in 95% ethanol and stored at – 20 °C for DNA extraction later.

DNA extraction, PCR and sequencing

Each individual insect sample was surface-sterilized using 3% NaOCl and rinsed three times with distilled water. Genomic DNA was extracted using the Isolate II genomic DNA Kit (Bioline, UK), following the manufacturer's instructions. The purity and concentration of the resultant extracted DNA was determined using Nanodrop 2000/2000c Spectrophotometer (Thermo Scientific). Polymerase chain reaction (PCR) was done to amplify the mitochondrial region and 2 sets of markers were used (Table 1). The PCR was carried out in a total reaction volume of 20 µL containing 5X My *Taq* Reaction Buffer (5 mM dNTPs, 15 mM $MgCl_2$, stabilizers and enhancers), 10 µmole of each primer, 0.5 mM $MgCl_2$, 0.25 µL My *Taq* DNA polymerase (Bioline, UK) and 15 ng/µL of DNA template. This reaction was set up in the Nexus Mastercycler gradient (Eppendorf). The following cycling conditions were used: initial denaturation for 2 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 45 sec annealing and 1 min at 72 °C, then a final elongation step of 10 min at 72 °C. The target gene region was 700 base pairs.

Table 1: Primer information used in this assay

Name	Sequence5' - 3'	Target	Source	Range	Annealing Temp (°C)
LepF1	ATTCAACCAATCATAAAGATATTGG	COI	Hajibabaei *et al*., 2006	Insects	52
LepR1	TAAACTTCTGGATGTCCAAAAAATCA	COI			
LCO1490	GGTCAACAAATCATAAAGATATTGG	COI	Folmer *et al*., 1994	Insects	50.6
HCO2198	TAAACTTCAGGGTGACCAAAAAATA	COI			

The amplified PCR products were resolved through a 1.2% agarose gel. DNA bands on the gel were analyzed and documented using KETA GL imaging system trans-illuminator (Wealtec Corp). Successively amplified products were excised and purified using Isolate II PCR and Gel Kit (Bioline, UK) following the manufacturer's instructions. The purified samples were shipped to Macrogen Inc Europe Laboratory, the Netherlands, for bi-directional sequencing.

The successful sequences were assembled and edited using Chromas Lite Version 2.1.1 (Thompson *et al*., 1997) and Geneious Version 8 (http://www.geneious.com) (Kearse *et al*., 2012). The primer sequences were identified and removed from the consensus sequences generated from both the forward and reverse reads. Phylogenetic and molecular evolutionary analyses for all the sets of sequences was inferred using MEGA 6, version 6.06 (Tamura *et al*., 2013) by the Maximum Likelihood method. The reliability of the tree was assessed using 1000 bootstrap replications.

DNA amplification and sequencing

DNA amplification by PCR was performed with the primers Ron and Nancy. The PCR conditions were as follows: 1x KAPA Robust Ready Mix (KAPA Biotech), 1x Enhancer A, 0.4 µM of each primer and 20 ng DNA. The PCR was performed in a verity PCR-cycler (Applied Biosystems) with the following conditions: 95°C for 5 min followed by 40 cycles of 95°C at 30 s, 55°C at 60 s and 72°C for 90 s and a final extension of 72°C for 10 min. Post-PCR purification was done using the NucleoFast Purification System (Separations). Sequencing was performed with each primer and Big Dye Terminator V1.3 (Applied Biosystems) followed by electrophoresis on the 3730xl DNA Analyser (Applied Biosystems). Sequences were analyzed using the Sequencing Analysis Version 5.3.1 software (Applied Biosystems). Editing of DNA sequences DNA sequences were manually edited (for base calling errors) pruned and aligned by ClustalW using the Bio Edit Sequence Alignment Editor (Hall, 1999) to create consensus sequences which were saved in the fasta format in MEGA5 (Hall, 1999).

Additionally, all consensus sequences were entered in BOLD to positively identify species. All specimen were identified to be from the same species, except two sample, which was identified to be from a different species, and was therefore used as an outgroup in the analysis. Additionally, the sequences were also exposed to Multiple Sequence Alignment by ClustalW (http://www.genome.jp/tools/clustalw/) to verify level of similarity between samples.

III. RESULTS

Summary of the identities of the processed samples

For conclusive identification of the species, similarity analysis was done. Similarity searches were conducted by querying the consensus sequences via BLAST at the GenBank database hosted by National Centre of Biotechnology Information (NCBI). BLAST (Basic Local Alignment Search Tool) algorithm finds regions of local similarity between sequences, in which consensus sequences were compared to reference sequences in the GenBank database. In addition to this, query was also done in BOLD (Barcode of Life Database).

Table 2.

Phyllum	Class	Order	Family	Genus	Species	Specimen similarity (%)
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100

Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	98
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	Tuta absoluta	90
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	95
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	Tuta absoluta	90
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	97
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100

Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	89
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	99
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100
Antropoda	Insecta	Lepidoptera	Aproaerema	Gelechiidae	simplexella	100

Identification by mtDNA (CO1) Based on comparisons with published sequences from the BOLD genebank, two samples were identified as possibly Tuta absoluta (Lepidoptera: Gelechiidae) (90% match), but the remaining samples (78) were identified as Aproaerma simplexella PS1 (Walker) (Lepidoptera: Gelechiidae) (89 to 100% match).There was very little genetic diversity within and between the specimens from the ten surveyed sites (Table 1).

IV. DISCUSSION

There has been a general assumption that the GLM occurring on groundnut in Africa has its origins in Asia,

with all reports from the African continent assuming the name A. modicella (Deventer) for the pest (Du Plessis, 2002, 2003). Contrary to this assumption, the mtDNA CO1 sequences of the GLM specimens examined in this study matched 100% with those of A. simplexella PS1 (previously Stomopteryx subsecivella (Ziller) (Bailey, 2007). This particular strain of A. simplexella is thought to be native to Australia where it is reported to be a pest for soybean (Common, 1990; Bailey, 2007). The evidence obtained from DNA analysis in the present study therefore suggests that the GLM in Uganda may have links with Australia. This is further supported by the fact that all GLM specimens taken from the ten widely separated sites in Uganda were identified as A. simplexella PS1, with A. modicella not listed in the most closely related species (Table 1). This infers that all infestations of GLM in Africa may be caused by the former, and not the latter species. Based on morphological characteristics, Shanower *et al.* (1993) suggested that the species found in Africa may be different from that found in India or Indonesia, describing the GLM in India as Anacampsis nerteria (Meyr.) (Meyrick, 1906), the one in Africa as Stomopteryx subsecivella and another in India-Indonesia as A. modicella (Deventer). It is thus clear that a large degree of uncertainty has always existed as to the correct classification of GLM in Africa. No attempt has however been made to discriminate between the species genetically. Previous to our DNA analysis, A. simplexella PS1 was known to be present only in Australia with recent identification in South Africa.

V. CONCLUSION

Mitochondrial DNA COI analysis identified GLM in Uganda as A. simplexella PS1 (100% match on the BOLD system), native to Australia, which suggested that Australia may be the origin of the pest. It is most likely that GLM being reported on groundnut in other parts of Africa is also A. simplexella PS1. Secondly, the 100% match on the BOLD system indicated that there was very little genetic diversity between and within the populations, suggesting that the pest might be from the same origin and could be a recent introduction to Uganda. Given that the sequences of GLM in Uganda matched those of A. simplexella PS1 and that the damage symptoms of the pest on groundnut are similar to those of A. modicella found in Asia, there is a need to determine if the two species are indeed genetically different. This has a bearing on the development and use of groundnut lines that are resistant to GLM, in countries where it is a problem. For the purpose of formulating strategies for managing the pest, there is also a need to determine its correct identity, its host range as well as it's in between season survival tactics in Africa.

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